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Alteration of heart uncoupling protein-2 mRNA regulated by sympathetic nerve and triiodothyronine during postnatal period in rats

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Abstract

To provide tissue-specific and developmental characteristics of gene expression of rat heart uncoupling protein-2 (UCP2), we investigated developmental alterations of UCPs mRNA expression in the heart and brown adipose tissue (BAT), and examined possible up-regulators of heart UCP2 expression using in vitro studies. Heart UCP2 mRNA expression was low during the early postnatal days followed by a rapid and significant increase in the 2nd postnatal week. Heart UCP3 mRNA remained undetectable until the 2nd postnatal week when the expression reached a small but significant peak. BAT UCP1 mRNA was abundantly expressed in the neonate, but the expression rapidly decreased to the adult level. The studies using cultured cardiomyocytes demonstrated that both 10^{-8} M triiodothyronine and 10^{-7} M isoproterenol, but not phenylephrine, increased UCP2 mRNA expression. These results indicate that the sympathetic nervous system and/or thyroid hormones may be involved in the up-regulation of heart UCP2 gene expression during postnatal development. The increase in postnatal heart UCP2 may provide a key link between the postnatal energy shift and adaptation of rat pups to their novel environment.

Keywords: Uncoupling protein-2; Development; Heart; β-Adrenoceptor; Triiodothyronine

1. Introduction

Uncoupling proteins (UCPs) are unique proteins located in the inner mitochondrial membrane. UCPs convert stored energy into heat [1,2] by transporting fatty acid anions to dissipate a proton electrochemical potential, uncoupled from ATP synthesis [3]. Indeed, UCPs play a potentially important role in determining metabolic efficiency. UCP1, the first uncoupling protein to be identified, is expressed exclusively in brown adipose tissue (BAT), an important site of energy expenditure in rodents [4]. In contrast to rodents, the abundance of BAT in large mammals is limited [5] and, therefore, UCP1 is not considered to be a significant regulator of human energy expenditure.

UCP2, cloned recently as a second member of the UCP family, is ubiquitously expressed in human and rodent tissues including heart [6,7]. Another novel member of the UCP family, UCP3, is preferentially expressed in skeletal muscle and BAT [8–10]. Thus, the new members, UCP2 and UCP3, are better suited for regulated thermogenesis in large mammals including humans. In contrast to rapidly emerging in-

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formation regarding synthesis and distribution, there have been a few studies on developmental implications of UCP2 and UCP3 [11,12]. However, the UCP2 gene has been reported to be expressed abundantly in the heart [6,8,10,13], leaving its physiological function in doubt.

By the 2nd week of the postnatal period, blood flow is changed from a fetal circulation pattern to a postnatal one in which an increasing work load on each ventricle results in a growing energy demand on the heart [14,15]. In fact, carnitine palmitoyltransferase I isoforms in cardiac mitochondria are known to change from a liver type to a muscle type in response to increased energy demand during postnatal development [16]. These findings led us to investigate whether heart UCP2 gene expression may be altered along with postnatal development and how the neonatal heart UCP2 expression is regulated.

To address these queries, we carried out the following studies. Firstly, on changes in gene expressions of heart UCPs during rat postnatal development. Secondly, we compared heart UCP2 mRNA expression with BAT UCP1 mRNA during rat postnatal development to determine whether heart UCP2 modulates thermogenetic activity similarly to BAT UCP1. Finally, we aimed to examine possible factors regulating rat heart UCP2 gene expression using cultured neonatal cardiac myocytes free from the effects of neural and humoral control systems.

2. Materials and methods

2.1. Animals

Wistar King A postnatal rat pups were used for developmental UCPs experiments. The dams were housed in a room with lighting from 07:00 to 19:00 h (a 12:12 h light:dark cycle) and maintained at 21 ± 1°C with humidity at 55 ± 5%. They were allowed free access to standard pelleted rat chow (CE-2, CLEA Japan, Tokyo) and tap water.

2.2. Procedures

The heart and BAT were surgically removed under ether anesthesia from four pups in each group consisting of the 2nd day and the 1st, 2nd, 3rd and 4th week of age. The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until use for analysis of UCP mRNAs. In the study on primary culture of cardiomyocytes, hearts taken from neonates on the 3rd day after birth were used. Cardiac myocytes were prepared by the modified Bollon's method, as described elsewhere [17].

2.3. Cell culture

The hearts removed from the neonatal pups were minced into 1-mm^3 pieces. The pieces were incubated in a 0.02% EDTA solution for 5 min at 37°C with shaking, and then in 5 ml of Hanks' balanced salt solution (HBSS) containing 3450 units collagenase (type IV; Cooper Biochemical, Philadelphia, PA) for 10 min in a 37°C water bath shaker at 120 rpm. The supernatant was discarded and the remaining pieces were treated with 5 ml of 1000 IU/ml Dispase (Godo Shusei, Tokyo) in Ca^{2+}- and Mg^{2+}-free HBSS using a magnetic stirrer for 20 min. The free-floating cardiac myocytes were centrifuged at $500 \times g$ for 5 min to collect the isolated cardiac myocytes. The myocytes resuspended in Dulbecco's modified Eagle medium (DMEM) (Nissui pharmaceutical, Tokyo) were seeded in plastic culture dishes and incubated for 90 min at 37°C in a CO_2 incubator with a gas phase of 5% CO_2 in air to exclude fibroblasts. 2×10^6 cells were planted in a 60-mm diameter culture dish containing 3 ml DMEM supplemented with 5% fetal bovine serum (Gibco Labs, Grand Island), 10 mmol/l HEPES and 100 IU kanamycin. After 48 h of culture to allow adherence of more than 70% of the cells to the culture dishes, cells were maintained in serum-free DMEM for 24 h. After this preconditioning period, the cultures were incubated in serum-free DMEM with the following agents or vehicles: isoproterenol (ISP), a β-adrenoceptor agonist; phenylephrine, an α-adrenoceptor agonist; and triiodothyronine (T_3) at concentrations of 10^{-7}, 10^{-7} and 10^{-8} M, respectively (Sigma Chemical, St. Louis, MO for each).

2.4. Northern blot analysis

Total RNAs were extracted using ISOGEN (Nippon Gene, Toyama) according to the manufacturer's protocol. Aliquots of 20 μg total RNA were electro-

phoresed on 1.2% formaldehyde-agarose gel, transferred onto a Biodyne B membrane (Pall Canada, Mississauga, Ont.) in 20×SSC solution by capillary blotting, and immobilized by exposure to ultraviolet light. Prehybridization and hybridization were carried out as previously described [18], using the ^{32}P-labeled cDNA fragments of rat UCP1, UCP2 and UCP3 as probes. Each cDNA probe was obtained by RT-PCR on total RNA isolated from Wistar King A rat BAT using oligonucleotide primers which have the following sequence. UCP1-sense primer, 5′-AGTGCCACTGTTGTCTTCAG-3′; UCP1-antisense primer, 5′-TTCTCCAAGTCGCCTATGTG-3′; UCP2-sense primer, 5′-CATCTTCTGGGAGGTAGC-3′; UCP2-antisense primer, 5′-AAGACAGGGCAGGAATGG-3′; UCP3-sense primer, 5′-GTTACCTTTCCACTGGACAC-3′; UCP3-antisense primer, 5′-CCGTTTCAGCTGCTCATAGG-3′. Membranes were washed with a solution of 2×SSC/0.1% sodium dodecylsulfate (SDS) at room temperature for 15 min and 1×SSC/0.1% SDS at 42°C for 15 min and 0.1×SSC/0.1% SDS at 65°C for 15 min. After washing the membranes, the hybridization signals were analyzed with a Bio-image analyzer (BAS 2000, Fuji Film Institution, Tokyo). The band intensities obtained by Northern blots were normalized to those of 18S ribosomal RNAs.

2.5. Statistical analysis

Values in each experiment were expressed as the mean ± S.E.M., unless otherwise specified. Developmental changes in UCPs were analyzed by one-way analysis of variance and Scheffé's F post hoc test. Unpaired Student's t-tests were used to assess the effects of drugs on UCP2 mRNA expression in cultured cardiomyocytes. P values less than 0.05 were defined as statistically significant.

3. Results

3.1. Developmental alteration of UCPs mRNA expression in the heart and BAT

As shown in Fig. 1, expression of UCP2 mRNA in the heart was low on the 2nd neonatal day, but gradually increased in the 1st postnatal week, and then rapidly increased to reach a plateau in the 2nd postnatal week. UCP2 expression at the plateau was greater than that on the 2nd day and in the 1st week by 207% and 108%, respectively ($P<0.05$ for each). The value in the 2nd week was sustained for the 3rd and 4th weeks, in which the values were larger than that on the 2nd day ($P<0.05$ for each). Unlike the expression of UCP2, UCP1 mRNA was undetectable in the heart at any postnatal stage tested. Heart UCP3 mRNA could not be detected until the 2nd postnatal week, when expression became detectable and was significantly greater than that in the 4th postnatal week ($P<0.05$) (Fig. 1).

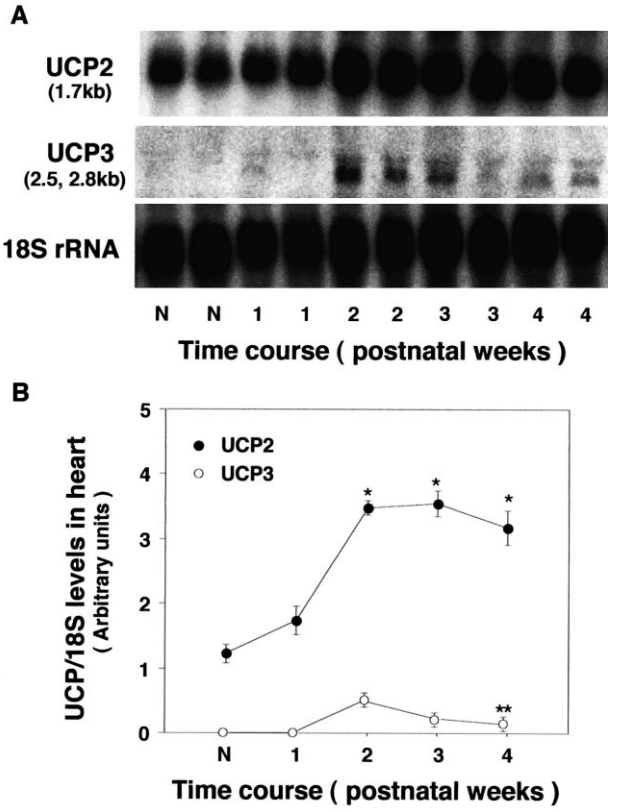

Fig. 1. Developmental alterations of UCP2 and UCP3 mRNA expression in the postnatal rat heart. (A) Representative Northern blots of the UCPs. Total RNAs (20 μg/lane) in various stages of life were used. (B) Time course alterations of the UCPs. Hybridizations with UCP2 and UCP3 cDNA probes were performed under the same condition. To quantify amounts of mRNAs from the heart, the blots were reprobed with 18S rRNA. Values and vertical bars, mean ± S.E.M. ($n=4$). *$P<0.05$ vs. the 2nd postnatal day, **$P<0.05$ vs. UCP3 in the 2nd postnatal week. N, the 2nd postnatal day; 1, the 1st postnatal week; 2, the 2nd postnatal week; 3, the 3rd postnatal week; 4, the 4th postnatal week.

UCP1 mRNA in BAT was abundantly expressed on the 2nd postnatal day, but the value decreased suddenly in the 1st postnatal week by 76% ($P < 0.05$). The lower value in the 1st week was maintained during the subsequent postnatal period, as illustrated in Fig. 2. Expression of UCP2 and UCP3 in BAT, similarly to UCP1, were greater in the early postnatal period than later. UCP3 mRNA decreased significantly in the 3rd and 4th postnatal weeks by 78% and 63% respectively compared with expression on the 2nd neonatal day ($P < 0.05$ for

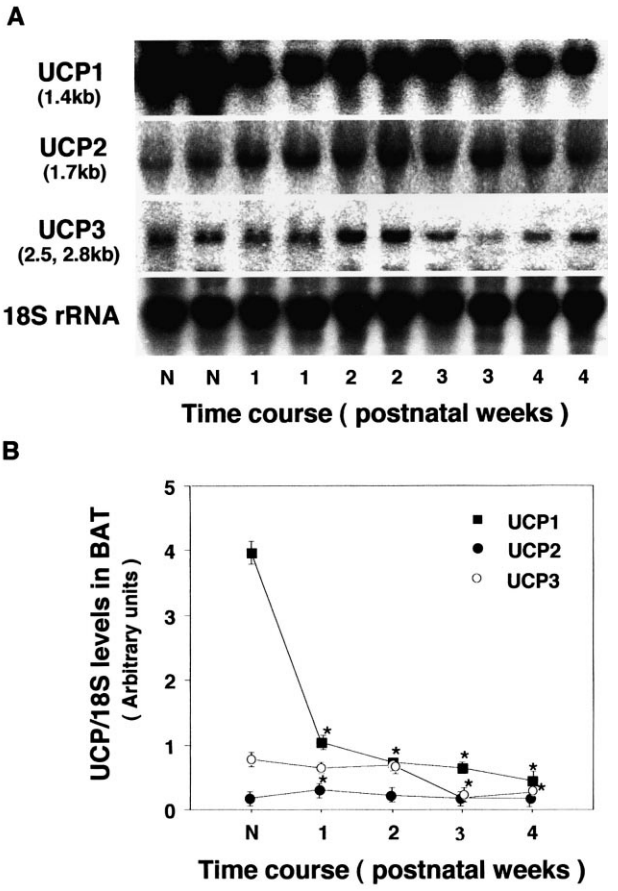

Fig. 2. Developmental alterations of UCP1, UCP2 and UCP3 mRNA expression in the postnatal rat brown adipose tissue. (A) Representative Northern blots of UCPs. Total RNAs (20 μg/lane) in various stages of life were used. (B) Time course alterations of the UCPs. Hybridizations with UCP1, UCP2 and UCP3 cDNA probes were performed under the same condition. To quantify amounts of mRNAs from BAT, the blots were reprobed with 18S rRNA. Values and vertical bars, mean ± S.E.M. ($n = 4$). *$P < 0.05$ vs. the 2nd postnatal day. N, the 2nd postnatal day; 1, the 1st postnatal week; 2, the 2nd postnatal week; 3, the 3rd postnatal week; 4, the 4th postnatal week.

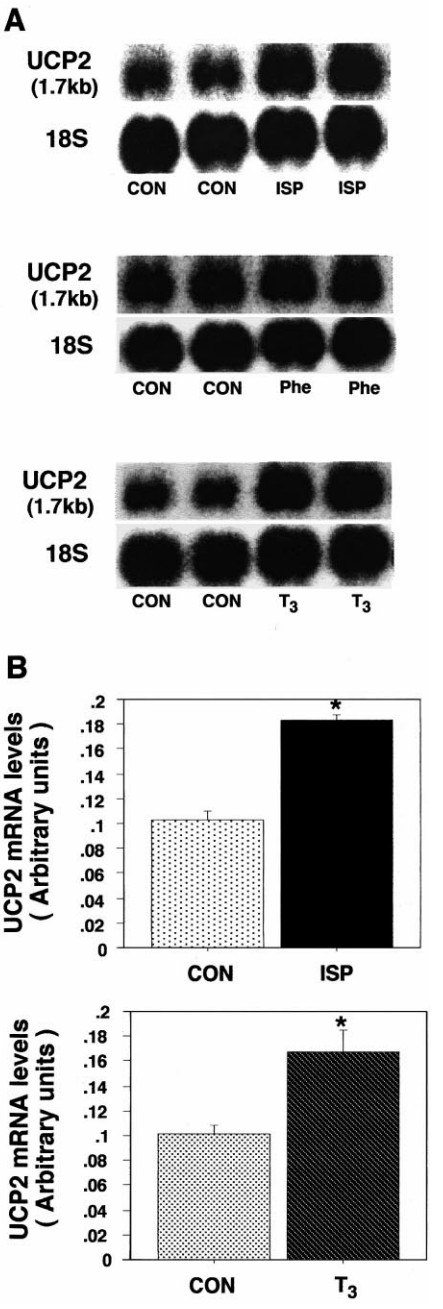

Fig. 3. Effects of isoproterenol (ISP), phenylephrine (Phe) and triiodothyronine (T_3) on UCP2 mRNA expression in neonatal cultured cardiomyocytes. Differences in loading were corrected by normalization using the corresponding 18S rRNA values. (A) Representative Northern blots of UCP2 after 24 h exposure to 10^{-7} M ISP (top panel), 10^{-7} M Phe (middle panel), 10^{-8} M T_3 (bottom panel). (B) The quantitative representations of the effects of ISP and T_3 on UCP2 mRNA in the cultured myocytes. Each column ($n = 4$ for each), the value of UCP2 mRNA expression. *$P < 0.05$ vs. the corresponding controls.

each), and UCP2 mRNA increased in the 1st postnatal week by 82% compared with the 2nd neonatal day ($P < 0.05$) (Fig. 2).

3.2. Adrenergic and hormonal effects on UCP2 mRNA expression in cultured cardiac myocytes

To explore up-regulating modulators of the heart UCP2 gene in the 2nd postnatal week, the effects of adrenoceptor agonists and T_3 on UCP2 mRNA expression were examined in cultured neonatal cardiac myocytes. Fig. 3 shows that UCP2 mRNA was increased at 24 h after addition of 10^{-7} M ISP ($P < 0.05$), but not after 10^{-7} M phenylephrine. Akin to ISP, addition of 10^{-8} M T_3 up-regulated UCP2 expression compared with the control DMEM treatment ($P < 0.05$).

4. Discussion

In the present study, UCP2 gene expression in the rat heart was predominantly up-regulated in the 2nd postnatal week and maintained the increased value throughout the experiment. In contrast to heart UCP2, UCP1 gene expression in BAT was already abundant on the 2nd day of the neonatal period. UCP1 gene expression in mouse BAT has been reported to reach to its peak at 15 h after birth, which suggests that the up-regulation of the UCP1 gene may be induced by thermal stress during transition from an intrauterine to an extrauterine environment [12]. The findings can be taken to indicate that the present demonstration of up-regulated BAT UCP1 in the neonatal period is consistent with mouse data, and may be caused by the thermal stress, as well. Up-regulation of the heart UCP2 gene, however, differed from that of BAT UCP1 in time to peak, while the present results from UCPs mRNA remain to be confirmed with Western blots. These findings indicate that heart UCP2 up-regulation may be unrelated to a sudden change in temperature of the environment. It can raise a question as to what mechanism(s) is (are) involved in the up-regulation of heart UCP2 gene expression in the postnatal period.

Recent reports showed that UCP2 and UCP3 mRNA expressions in skeletal muscle were up-regulated in response to the increase of plasma fatty acids levels [19,20]. Energy substrates used by the heart has been known to change after the birth. In the fetal and neonatal periods, the heart utilizes lactate and glucose as its main energy substrates, while in the adult, fatty acids are the main energy substrate. This energy shift depends on the following factors. First, increasing work load on the heart partly induced by thyroid hormones and sympathetic innervation. Second, changes of metabolic enzymes involved in carbohydrate and fatty acid utilization. Third, an increase in circulating fatty acids levels. Fourth, maturation of mitochondrial processes including activities of tricarboxylic acid cycle and electron transport chain enzymes [21]. These reports suggest the possibility that UCP2 mRNA up-regulation during development could be due to the increase of fatty acids uptake into the heart. Fatty acid is known to bind to peroxisome proliferator-activated receptor (PPAR) as a potent ligand [22,23]. UCP1 gene has been reported to contain PPAR response element [24] and UCP2 is up-regulated by PPAR agonist in white adipose tissue [25]. Taken together, it is likely that fatty acids may work as a ligand for PPAR to promote transcription of UCP2 in the postnatal rat heart. In the present study, however, UCP2 was up-regulated in response to T_3 and ISP in serum free in vitro experiments. The possible existence of thyroid hormone response element and cAMP response element in the UCP2 gene could provide the direct effect of T_3 and ISP on transcription of UCP2. In fact, thyroid hormones increase rapidly between the 8th and the 15th postnatal days [26], and sympathetic innervation of the heart becomes fully developed by the 3rd week of the postnatal period [27,28]. As another possibility, UCP2 could be associated with apoptosis. UCP2 was reported to decrease generation of hydrogen peroxide (H_2O_2) in mitochondria [29]. H_2O_2 has been found to induce apoptosis in the cultured cardiomyocytes [30]. While direct evidence is lacking at present, these data are compatible with the possibility that UCP2 may play a crucial role in protection against apoptosis during heart development. In fact, apoptosis is active in the neonatal period and decreases sharply by 21 postnatal days [31].

In summary, developmental studies on UCPs gene expression demonstrated that heart UCP2 gene expression increased to a peak in the 2nd postnatal

week. Both ISP and T_3 up-regulated heart UCP2 mRNA in neonatal cultured cardiomyocytes. These results indicate that the sympathetic nervous system and/or thyroid hormones could be involved in the up-regulation of heart UCP2 gene expression during postnatal development. The increase in postnatal heart UCP2 may provide a key link between the postnatal energy shift and adaptation of rat pups to their novel environment.

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